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(54) Antihypercholesteraemic Agent, Monacolin K, and Its Preparation

(57) A new compound which we refer to as Monacolin K, has the molecular formula $C_{24}H_{38}O_5$ and has been found to have valuable antihypercholesteraemic activity. It can be produced by cultivating suitable micro-organisms from the genus *Monascus*, especially *Monascus ruber* strain 1005 (FERM 4822).

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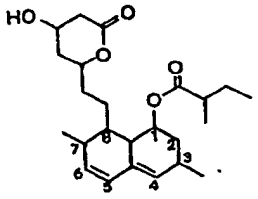
SPECIFICATION

Antihypercholesteraemic Agent, Monacolin K, and its Preparation

The present invention relates to a new compound having antihypercholesteraemic activity and which we have named Monacolin K. Monacolin K can be produced by cultivating various microorganisms of the genus *Monascus*.

Thus the present invention consists in a compound, Monacolin K, having the formula:



The invention further consists in a process for preparing an antihypercholesteraemic agent designated Monacolin K, which comprises cultivating a Monacolin K-producing microorganism of the genus *Monascus* in a culture medium therefor.

The invention still further consists in a pharmaceutical composition comprising Monacolin K in admixture with a pharmaceutically acceptable carrier or diluent.

High blood cholesterol levels are recognized as being one of the main causes of cardiopathy, such as cardiac infarction or arteriosclerosis. As a result, considerable research has been undertaken with a view to discovering physiologically acceptable substances which are capable of inhibiting cholesterol biosynthesis and thus reducing blood cholesterol levels. One such compound is ML-236, which forms the subject of our United Kingdom Patent Specification No. 1,453,425. ML-236 is produced by cultivating microorganisms of the genus *Penicillium*.

On investigating fungi of the genus *Monascus*, it was found that these, particularly *Monascus ruber* strain 1005 (FERM 4822), produced an antihypercholesteraemic agent having substantially better activity than that of ML-236. This agent was named Monacolin K.

All microorganisms of the genus *Monascus* which are capable of producing Monacolin K may be employed in the process of the present invention. Especially useful are strains of *Monascus ruber*, particularly *Monascus ruber* strain 1005 (FERM 4822).

Monascus ruber strain 1005 (FERM 4822) is a newly isolated microorganism having the following microbiological properties. It was isolated from foodstuffs produced in Thailand and deposited on 16 February 1979 under the accession No. FERM 4822 with the Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Japan and under the accession No. NRRL 12073 with the Agricultural Research Service, Northern Regional Research Laboratory, USA.

1. *Growth*

The growth on a potato-glucose-agar medium at 25°C is fast and the diameter of the colony reaches 6—6.5 centimetres 10 days after inoculation. The colony is flat and a relatively thin basal layer of hyphae develops. Development of aerial hyphae is poor; the aerial hyphae are white and most of them are woolly. Many cleistothecia are formed on the basal layer of hyphae and turn reddish-brown on maturity. Both the surface and the reverse of the colony are brown to reddish-brown in colour.

The growth on Sabouraud's agar medium at 25°C is very fast and the diameter of the colony reaches 6—6.5 centimetres 10 days after inoculation. The surface of the colony is very flat, and basal hyphae and aerial hyphae develop better than on potato-glucose-agar medium. Cleistothecia counts are very few. The surface of the colony is reddish-yellow to reddish-brown in colour and the reverse is reddish-brown to dark brown.

The growth on oatmeal agar at 25°C is slow and the diameter of the colony reaches 1.5—2 centimetres 10 days after inoculation. The colony is flat. Development of aerial hyphae and formation of cleistothecia are both very poor. Both the surface and the reverse of the colony are dark red to reddish-brown in colour.

The growth on Czapek's agar medium at 25°C is very slow and the diameter of the colony reaches 1.6—1.8 centimetres 10 days after inoculation.

The rates of growth on each of the above media at 37°C are substantially equal to those at 25°C.

2. *Morphological Properties*

The cleistothecia are spherical and 30—60 microns in diameter; their walls are thin and membranous; their stalks have septal walls and each consists of a hypha of diameter 3.5—4.5 microns and length 15—80 microns. The ascus consists of 8 spores and is nearly spherical and evanescent. The ascospores are colourless and ovoid or ellipsoid; they have a size of 4—5×4—7 microns; and their surfaces are smooth. The conidia are colourless and spherical or pyriform; their size is 6—9×6—11 microns; their bases are truncate and their walls are relatively thick and smooth. The conidia are linked basipetally as a type of meristum arthrospore. The conidiophore is like a vegetative hypha and is branched or unbranched; the conidia being formed at the top. The mycelia are colourless and branched and have septal walls; most of them have a diameter of 3—5 microns.

Based on the observations of its characteristics as reported above, this microorganism was identified as a strain of *Monascus ruber* van Tieghem.

Microbiological properties of *Monascus ruber*

have been reported in the following literature: Takada, Transactions of the Micological Society of Japan, *9*, 125—130 (1969) [Materials for the Fungus Flora of Japan (7)]; and van Tieghem, Bull. Soc. Botan. France, *31*, 227 (1884). Ascospore generation of the strain has been reported by Cole *et al* in the Canadian Journal of Botany, *46*, 987 (1968), "Conidium Ontogeny in hyphomycetes. The imperfect state of *Monascus ruber* and its meristum arthrospores".

Although the use of *Monascus ruber* strain 1005 is hereafter specifically exemplified, it will be appreciated that any strains of the genus *Monascus*, including varieties and mutants, which are capable of producing Monacolin K can be used in the process of the invention.

Monacolin K may be produced by cultivating the chosen microorganism in a culture broth under aerobic conditions, using the same techniques as are well known in the art for the cultivation of fungi and other microorganisms. For example, the Monacolin K-producing microorganism may first be cultivated on a suitable medium and then the produced microorganisms may be collected and inoculated into and cultivated on another culture medium to produce the desired Monacolin K; the culture media used for multiplication of the microorganism and for production of Monacolin K may be the same or different.

Any culture medium well known in the art for the cultivation of fungi may be employed, provided that it contains, as is well known, the necessary nutrient materials, especially an assimilable carbon source and an assimilable nitrogen source. Examples of suitable sources of assimilable carbon are glucose, maltose, dextrin, starch, lactose, sucrose and glycerine. Of these sources, glucose, glycerine and starch are particularly preferred for the production of Monacolin K. Examples of suitable sources of assimilable nitrogen are peptone, meat extracts, yeast, yeast extract, soybean meal, peanut meal, corn steep liquor, rice bran and inorganic nitrogen sources. Of these nitrogen sources, peptone is particularly preferred. When producing Monacolin K, an inorganic salt and/or a metal salt may, if necessary, be added to the culture medium. Furthermore, if necessary, a minor amount of a heavy metal may also be added.

The microorganism is preferably cultivated under aerobic conditions using cultivation methods well known in the art, for example solid culture, shaken culture or culture under aeration and agitation. The microorganism will grow over a wide temperature range, e.g. from 7 to 40°C, but, especially for the production of Monacolin K, the more preferred cultivation temperature is within the range from 20 to 35°C.

During the cultivation of the microorganism, the production of Monacolin K may be monitored by sampling the culture medium and measuring the physiological activity of the Monacolin K in the culture medium by the test described hereafter. Cultivation may then be continued until a substantial accumulation of Monacolin K has been achieved in the culture medium, at which time the Monacolin K may be isolated and recovered from the culture broth by any suitable combination of isolation techniques chosen having regard to its physical and chemical properties. For example, any or all of the following isolation techniques may be employed: extraction of the liquor from the culture broth with a hydrophilic solvent (for example, diethyl ether, ethyl acetate, chloroform or benzene); extraction of the organism with a hydrophilic solvent (such as acetone or an alcihol); concentration; dissolution into a more polar solvent (e.g. acetone or an alcohol); removal of impurities with a less polar solvent (such as petroleum ether or hexane); gel filtration through a column of a material such as Sephadex (a trade name for a material available from Pharmacia, Co., Ltd., U.S.A.); absorptive chromatography with active carbon or silica gel; and so on. By using a suitable combination of these techniques, the desired Monacolin K can be isolated from the culture broth as a pure substance.

Monacolin K was found to have the following properties:

1. Colour and form:
   Colourless crystals.
2. Melting point:
   157—159°C (with decomposition).
3. Elemental analysis:
   C, 71.56%; H, 8.85%; O, 19.59%.
4. Molecular weight:
   404 (by mass spectrometry).
5. Molecular formula:
   $C_{24}H_{36}O_5$.
6. Ultraviolet absorption spectrum (methanol):
   As shown in Figure 1 of the accompanying drawings having maxima at 232, 238 and 246 mμ.
7. Infrared absorption spectrum (KBr):
   As shown in Figure 2 of the accompanying drawings.
8. Nuclear magnetic resonance spectrum (60 MHz proton):
   As shown in Figure 3 of the accompanying drawings in deuterated chloroform, using tetramethylsilane as internal standard.
9. Nuclear magnetic resonance spectrum (^{13}C):
   As shown in Figure 4 of the accompanying drawings, in deuterated methanol.
10. Solubility:
    Soluble in lower alcohols (e.g. methanol, ethanol and propanol), acetone, chloroform, ethyl acetate and benzene. Insoluble in petroleum ether and hexane.
11. Specific rotation:
    $[\alpha]_D^{26}=+307.6$(c=1, methanol).
12. Thin layer chromatography:
    Rf=0.47 [No. 5715 Kieselgel $60F_{254}$ silica gel)Merck & Co., Ltd.) developed by a 4:1 volume mixture of methylene chloride and acetone, detectable as an ultraviolet radiation-absorbing lump, 50% v/v sulphuric acid (a pale red to reddish-brown colour

develops on heating) or with iodine].

The compound is neutral and is insoluble in neutral or acidic aqueous media. It is converted to an acidic substance upon treatment with an alkali and can then be dissolved in water. This acidic substance can be extracted with ethyl acetate or chloroform at an acid pH value and will revert to Monacolin K on evaporation of the solvent.

The physiological activity of Monacolin K can be assayed and determined quantitatively by the following *in vivo* test.

In vivo test with rabbits

In this test, the ability of Monacolin K to reduce cholesterol levels in rabbit blood is measured. The animals employed should weigh from 2.5 to 3.0 kg. Immediately prior to starting the test, blood is collected from the vein in an ear of each rabbit and the cholesterol level in the blood serum is measured by a conventional method. A predetermined quantity of Monacolin K is then administered orally continuously for 1 to 5 days and the cholesterol level in the blood serum after administration is measured. The potency of the Monacolin K or Monacolin K-containing culture medium can be determined quantitatively from the cholesterol values obtained prior to and after administration of Monacolin K.

We have demonstrated the ability of Monacolin K to lower the blood and liver cholesterol levels by varous *in vivo* tests.

Reduction of blood cholesterol levels in rats

The animals used were rats of the Wistar Imamichi strain, each having a body weight of about 300 g. The rests were conducted on groups of rats, each group consisting of 5 animals. Each animal was intraveneously injected with 400 mg/kg of Triton WR-1339 (a trade name for a material known to increase the blood cholesterol level) whilst simultaneously administering intraperitoneally 10 mg/kg of Monacolin K. 14 hours after intraperitoneal administration, the rats were sacrificed by bleeding and the blood was collected and its cholesterol level was determined by conventional means. As a result, it was established that blood cholesterol levels had been reduced, as compared with a control group of animals to which Triton WR-1339 alone had been administered, by 23.9%.

Reduction of blood cholesterol levels in rabbits

The test animals used were rabbits having a body weight of from 2.7 kg to 2.9 kg. Each rabbit was given orally 1 mg/kg of Monacolin K twice each day (morning and evening) continuously for 5 days. Prior to administration and at 3 and 5 days after administration, blood was collected from a vein in the ear and the cholesterol levels in the blood serum were determined. As a result it was found that the cholesterol levels at 3 and 5 days after administration of Monacolin K were 15% and 29%, respectively, lower than the level prior to administration of Monacolin K.

In addition to its valuable inhibitory effect on the biosynthesis of cholesterol, Monacolin K has a very low toxicity. Thus, the acute oral toxicity (LD_{50}) of Monacolin K in the mouse is 1 g/kg body weight or more.

The Monacolin K may be administered orally or parenterally in the form of a capsule, tablet, injectable preparation or any other known formulation, although we normally prefer to administer it orally. The dose will vary, depending upon the age and body weight of the patient and the severity of the condition, but, in general, the daily dose for an adult would be from 0.5 to 50 mg, either as a single dose or in 2 or 3 divided doses. However, in view of the low toxicity of the compound, higher doses may be employed if required.

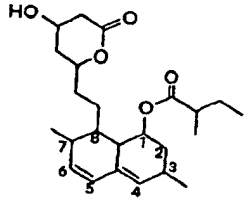
The invention is further illustrated by the following non-limiting Example.

Example

Monascus ruber 1005 strain was inoculated onto a liquid culture medium containing 6% w/v glucose, 2.5% w/v peptone, 0.5% w/v corn steep liquor and 0.5% w/v ammonium chloride. Cultivation was continued under aerobic conditions at a temperature of 28°C for 10 days. The resulting filtrate (5 litres) of the culture broth was adjusted to a pH value of 3 by the addition of 6N hydrochloric acid and then extracted with an equal volume of ethyl acetate. The solvent was evaporated under reduced pressure from the extract and the resulting residue was dissolved in 100 ml of benzene. Insolubles were filtered off. The filtrate was washed twice, each time with 100 ml of a 5% w/v aqueous solution of sodium bicarbonate. 100 ml of a 0.2 N aqueous solution of sodium hydroxide were then added to the washed filtrate and the mixture was stirred at room temperature. After confirming the disappearance of Monacolin K from the benzene layer by thin layer chromatography, the aqueous layer was separated off. The pH value of the aqueous layer was then adjusted to 3 by addition of 6N hydrochloric acid and the resulting solution was extracted twice, each time with 100 ml of ethyl acetate. The extract was evaporated to dryness under reduced pressure, giving 260 mg of an oil. This oil was dissolved in benzene and allowed to crystallize and then recrystallized from an aqueous acetone solution to give 87 mg of colourless needles of Monacolin K having the properties heretofor described.

Claims

1. A compound of formula:



2. A process for preparing an antihypercholesteraemic agent designated Monacolin K, which comprises cultivating a Monacolin K-producing microorganism of the genus *Monascus* in a culture medium therefor.

3. A process according to Claim 2, in which said microorganism is a strain of *Monascus ruber*.

4. A process according to Claim 3, in which said strain is *Monascus ruber* strain 1005 (FERM 4822).

5. A process according to any one of Claims 2 to 4, in which cultivation is carried out at a temperature of from 7 to 40°C.

6. A process according to Claim 5, in which said temperature is from 20 to 35°C.

7. A process according to Claim 2, substantially as hereinbefore described with reference to the foregoing Example.

8. Monacolin K when produced by a process according to any one of Claims 2 to 7.

9. A pharmaceutical composition comprising a compound according to Claim 1 or Claim 8 in admixture with a pharmaceutically acceptable carrier or diluent.

10. A composition according to Claim 9, in a form suitable for oral or parenteral administration.

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FIG. 1

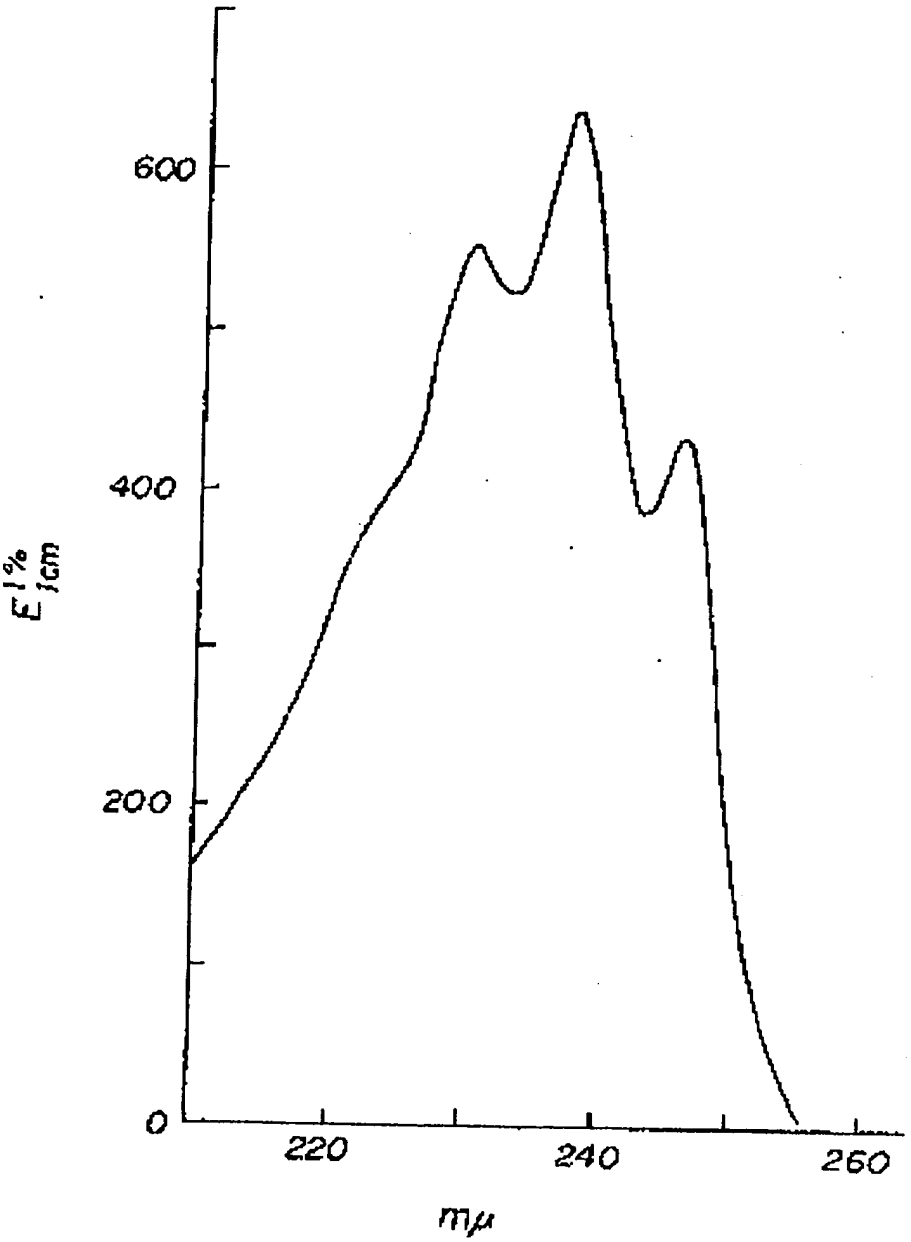

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FIG. 2

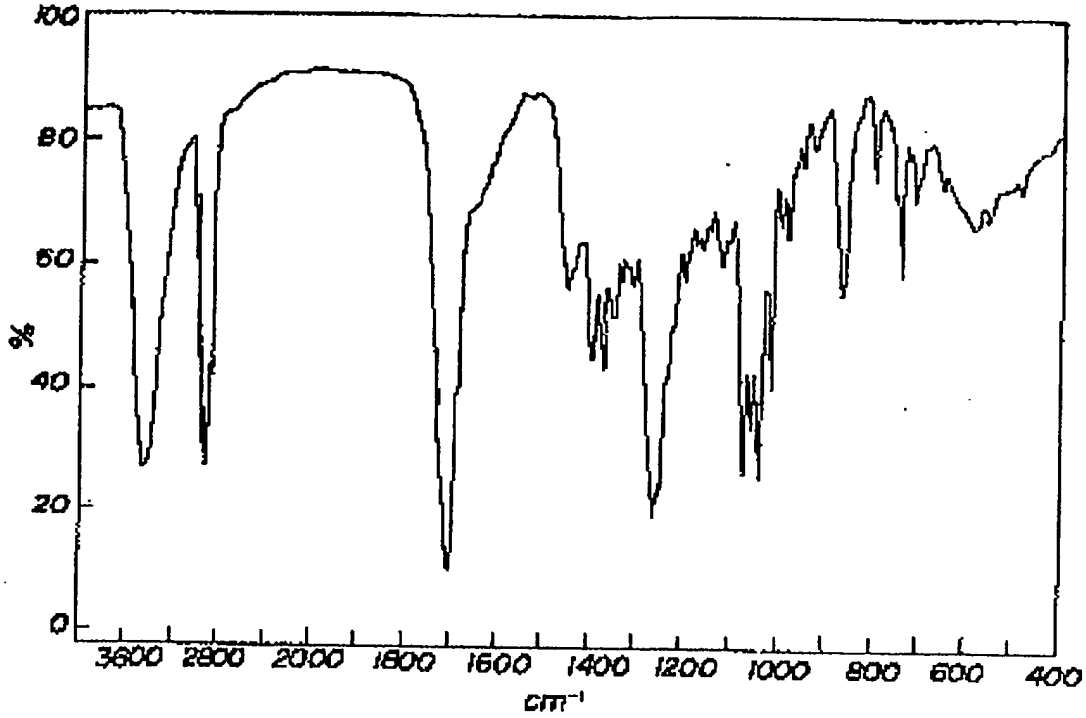

FIG. 3

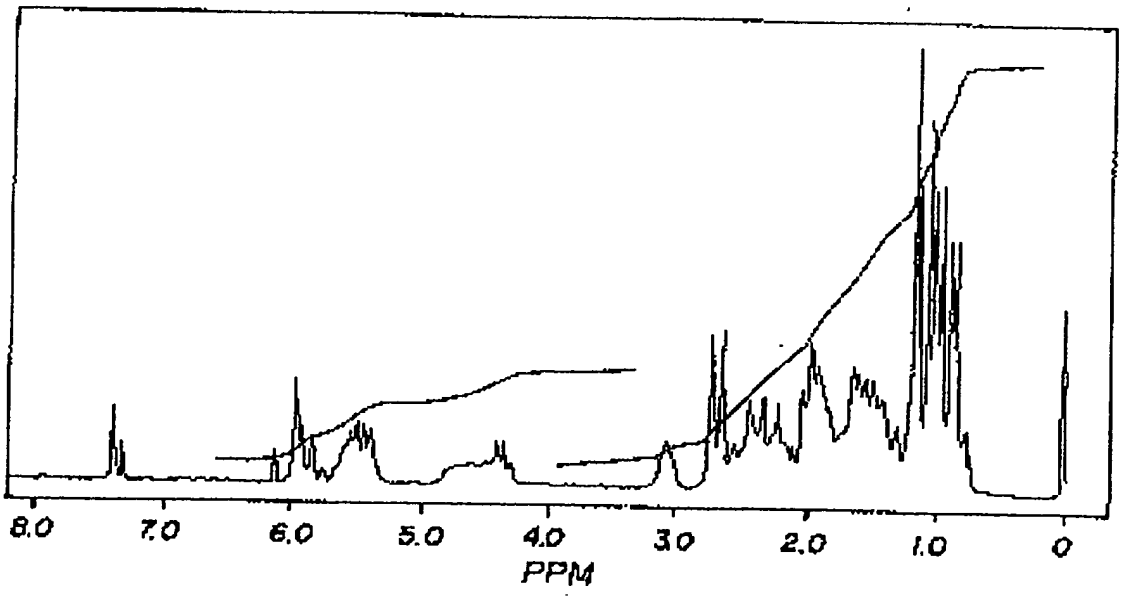

FIG. 4